Association of homologous chromosomes during floral development

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Reduction in chromosome number and genetic recombination during meiosis require the prior association of homologous chromosomes, and this has been assumed to be a central event in meiosis. Various studies have suggested, however, that while the reduction division of meiosis is a universally conserved process, the pre-meiotic association of homologues differs among organisms. In the fruit fly *Drosophila melanogaster*, some somatic tissues also show association of homologues [1,2]. In the budding yeast *Saccharomyces cerevisiae*, there is some evidence for homologue association during the interphase before meiotic division [3,4], and it has been argued that such associations lead directly to meiotic homologue pairing during prophase I [5]. The available evidence for mammals suggests that homologous chromosomes do not associate in germ cells prior to meiotic prophase [6]. To study the occurrence of homologue pairing in wheat, we have used vibratome tissue sections of wheat florets to determine the location of homologous chromosomes, centromeres and telomeres in different cell types of developing anthers. Fluorescence *in situ* hybridization followed by confocal microscopy demonstrated that homologous chromosomes associate pre-meiotically in meiocytes (germ-line cells). Surprisingly, association of homologues was observed simultaneously in all the surrounding somatic tapetum cells. Homologues failed to associate at equivalent stages in a homologue recognition mutant. These results demonstrate that the factors responsible for the recognition and association of homologues in wheat act before the onset of meiotic prophase. The observation of homologue association in somatic tapetum cells demonstrates that this process and meiotic division are separable.

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Results and discussion

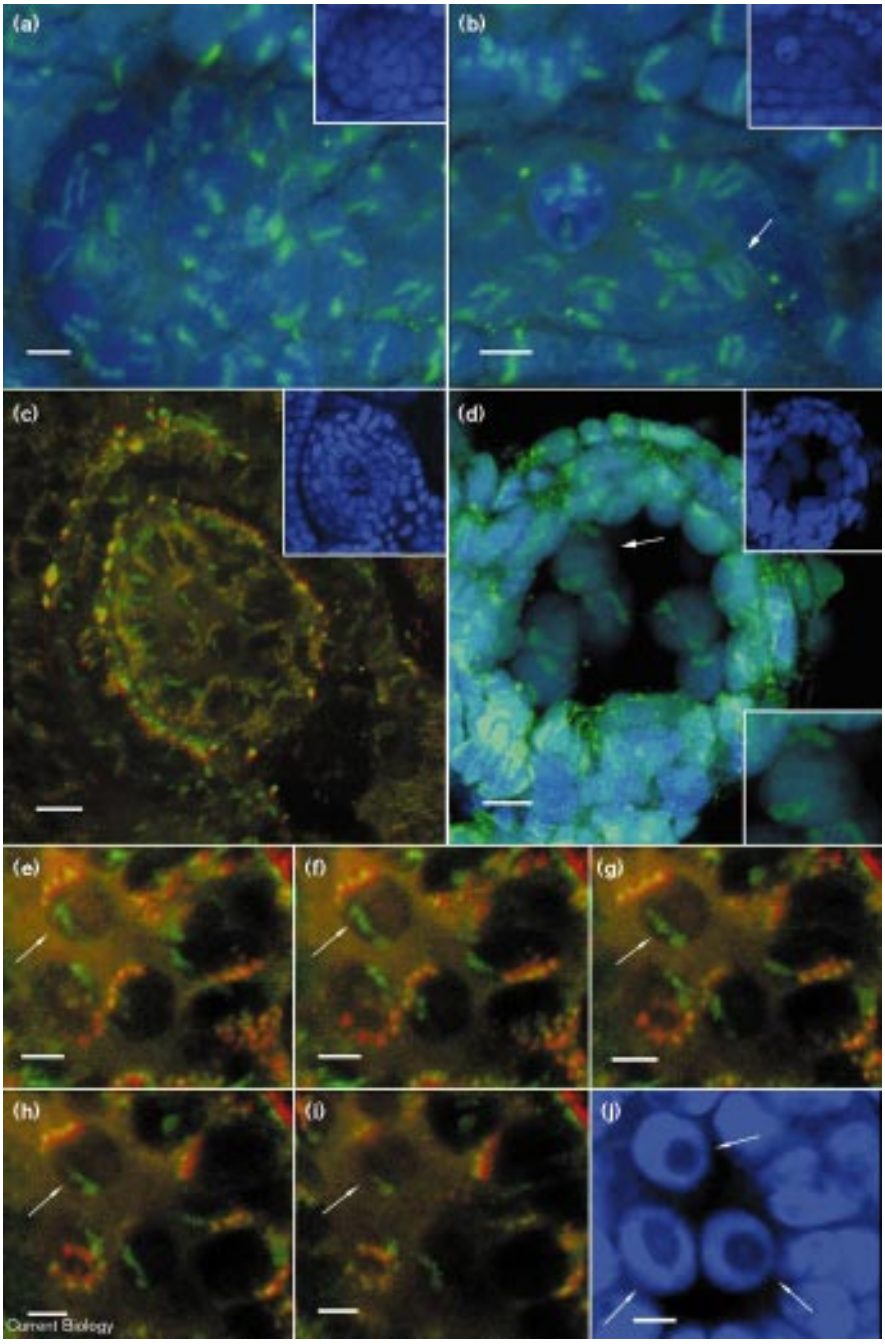
In multicellular organisms, attempts to investigate the chromosomal events occurring in the interphase preceding meiosis have encountered difficulties because of the lack of specific landmarks and time points during this period. Most previous studies on plants have dissected anthers and spread or squashed cells for analysis. The loss of cellular and tissue organisation makes it difficult to distinguish between developing meiocytes prior to meiotic prophase and surrounding somatic cells in these preparations.

To investigate pre-meiotic events, we have studied the anthers from immature male flowers from a line of wheat which has a pair of homologous barley chromosomes substituted for a pair of wheat chromosomes. The barley homologues were visualized by genomic *in situ* hybridization. A vibratome was used to cut sections 100 μm thick (representing two to three cell layers) from florets, which gives good preservation of tissue and cellular structure. Confocal microscopy is necessary to produce clear images of these thick sections. Time points can be set using consecutive florets (flowers) within the same inflorescence, and cell types in the anther are easily identified on the basis of their morphology [7]. By the use of fluoresence *in situ* hybridization (FISH) and confocal microscopy, three-dimensional reconstructions of the positions of centromeres, telomeres and the pair of introduced chromosomes can be made in all the cell types. Well characterised meiotic mutants are also available [8,9], some of which have been shown to affect chromatin structure at the centromeres [10].

More than 50% of angiosperms are polyploid, and an additional level of genetic control of the pairing of homo-logues has evolved. Hexaploid wheat, the subject of the present study, possesses a well characterised gene locus, the *Ph1* (*Pairing homoeologous 1*) locus, controlling the pairing behaviour of homologous and homoeologous chromosomes [8,9]. We demonstrate here that in polyploid wheat the homologues associate in pre-meiotic interphase, and that deletion of the *Ph1* locus disrupts this association.

The developmental stage of the anthers was identified using three criteria. First, we used younger florets from the same inflorescence as those staged at leptotene through traditional squashing methods. Second, we used meiocyte morphology (nuclear appearance and nucleoli numbers and positioning) as defined by Bennett *et al.* [7]. Third, we analysed centromere positions. Throughout pre-meiotic interphase centromeres are clustered at a position in the opposite nuclear pole to telomeres. A ‘clustering switch’, at which the centromeres separate, chromatin reorganizes

Figure 1

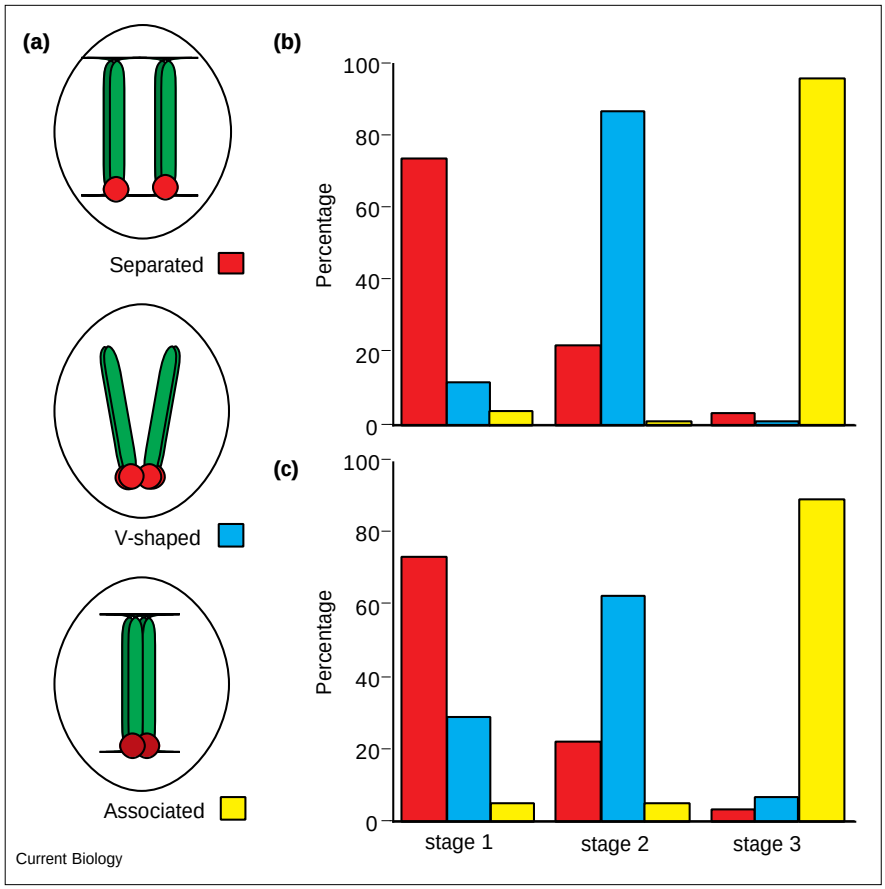


Fluorescence *in situ* hybridization (FISH) to wheat tissue sections. Colour overlays of confocal images from 100 μm thick sections of florets. Sections were labelled by FISH using probes to centromeres and total barley genomic DNA. Sections were counterstained with DAPI (4′,6-diamidino-2-phenyl-indole) to show the total chromatin. The data were collected using a BioRad MRC-1024 UV confocal microscope. The chromosomes are shown in green, centromeres in red/orange and chromatin in blue [top right inset in (a)–(d)]. Images in (a)–(d) were made by projecting several optical sections representing approximately 15–20 μm in depth, equivalent to one cell thickness. This was necessary to show both labelled chromosomes for the nuclei of interest, as the orientation of the chromosomes rarely coincides with the plane of optical sectioning. Our analysis and conclusions were made from studying the entire 3-D confocal section stacks, typically containing three cell layers. However, it is not possible to publish these in their entirety. Projections such as these are complicated by a number of factors: the focal planes chosen for projection will not only include whole nuclei, but also will include parts of neighbouring nuclei lying above and below those of interest. This is particularly the case for tapetal cells, which have irregular shapes and are densely packed. **(a)** Early stage sporangium. The cell types are not morphologically distinguishable, and the homologues are mostly separated. Scale bar = 10 μm. **(b)** Later stage sporangium. Note the V-shaped appearance (arrow) of homologues in many nuclei. Scale bar = 10 μm. **(c)** Sporangium prior to the onset of prophase I. Note centromere clustering (orange) and homologue association (green) in the meiocytes and in the tapetum. Scale bar = 15 μm. **(d)** *Ph1*-deficient sporangium at late pre-meiotic interphase. Note that the homologues are not associated (arrow and bottom inset). Scale bar = 10 μm. **(e–i)** Five consecutive confocal sections [enlarged from the data set shown in projection in (c)]. One pair of associated homologues is indicated by an arrow in each section. Spacing between the sections 1.5 μm. Scale bar = 5 μm. **(j)** Projection of DAPI image for sections shown in (e–i). This portion of the data set includes three meiocytes (arrows). Scale bar = 5 μm.

and telomeres aggregate, correlates with the first stage of meiotic prophase I (L.A-A, P.S. and G.M., unpublished observations). Here we present data on stages prior to leptotene.

The developing meiocytes and the surrounding tapetum cells (somatic cells physiologically important for the nutrition and development of the germ cells) were morphologically indistinguishable 3–5 days prior to meiotic prophase (Figure 1a). Homologous chromosomes showed no significant association at this stage, termed stage 1 (Figure 1a and the ‘separated’ category in Figure 2a). As development progressed (1–3 days before meiosis, termed stage 2) and meiocytes and tapetum became distinguishable by their positions, the two homologues often appeared as a single characteristic V-shaped structure (Figure 1b and the ‘V-shaped’ category in Figure 2a) with centromeres located at the vertex of the V. Centromeres in developing meiocytes

Figure 2



Dynamics of homologue configuration in the nucleus. (a) Schematic diagram of the configurations analysed. Top, separated represents individual chromosomes in a Rabl configuration [12], with centromeres at one end and telomeres at the opposite end. Centre, V-shaped represents two homologues in a single V with centromeres located at its vertex. Bottom, associated represents the two homologues with centromeres at one end and telomeres at the opposite end. Centromeres are shown in red. (b,c) Relative percentages (from Table 1) of the three categories for each of the three stages defined in the text for meiocytes (b) and tapetum cells (c).

and somatic interphase cells have been shown to associate in pairs [10]. The nature of these associations could not be assessed, however, without homologue-specific centromere probes. Following the last mitotic division of the meiocytes, a distinct somatic cell layer (the tapetum layer) can be observed surrounding the meiocytes (Figure 1c).

Table 1

Numbers and relative percentages of nuclei analysed.

	Meiocytes			Tapetum cells			Undifferentiated			Total
	Separated	V-shaped	Associated	Separated	V-shaped	Associated	Separated	V-shaped	Associated	no. of nuclei
Stage 1	NA	NA	NA	NA	NA	NA	97 (72%)	31 (23%)	6 (5%)	134
Stage 2	4 (10%)	36 (88%)	1 (2%)	39 (31%)	77 (61%)	10 (8%)	NA	NA	NA	167 (41 meiocytes; 126 tapetum)
Stage 3	3 (5%)	1 (2%)	57 (93%)	8 (6%)	11 (9%)	109 (85%)	NA	NA	NA	189 (61 meiocytes; 128 tapetum)
Ph1 mutant stage 3	22 (76%)	7* (24%)	0	37 (55%)	25* (37%)	5 (8%)	NA	NA	NA	96 (29 meiocytes; 67 tapetum)

NA, not applicable. *Unlike the wild type the barley homologues were observed in contact at the centromere or another site in the Ph1 mutant. For simplicity these have been categorized as V-shaped.

During the pre-meiotic interphase, meiocytes show a characteristic nuclear appearance with a single nucleolus at the nuclear periphery [7] (Figure 1c,j). At this stage (termed stage 3) homologous chromosomes showed complete association in most meiocytes (Figure 1c,e–j and the 'associated' category in Figure 2a) and tapetum cells, while centromeres remained clustered (Figure 1c).

In order to quantify these observations, a total of 586 nuclei (Table 1) from 29 anther sections were scored and categorized as shown in Figure 2. The 'separated' category showed two completely separate fluorescent lines corresponding to the homologues; the 'V-shaped' category showed two fluorescent lines associated at one end; and the 'associated' category showed a single fluorescent line corresponding to the two homologues. Nuclei were only included in the analysis if they were clearly separated from neighbouring nuclei, if the FISH signals were clearly visible and if the nucleus was visible throughout its entire depth. At stage 1, 72% of nuclei had separated homologues. At stage 2, 88% of meiocytes and 61% of tapetum cells had V-shaped homologue configurations. At stage 3, 93% of meiocytes and 85% of tapetum cells had associated homologues (Figure 2).

A similar wheat substitution line also deficient for the *Ph1* locus was used to investigate the effect of this locus on homologue organization during anther development. In the absence of the *Ph1* locus, pairing can occur not only between homologues but also between equivalent chromosomes from the three different constituent genomes (homoeologues) [8]. The anther and nuclear morphology of this mutant differs from the wild type, but apart from nuclear morphology, the other staging criteria used for the wild type were used independently to stage the mutant. An image of an anther in stage 3 from this line is shown in Figure 1d. Unlike the wild type, there was no association of the barley homologues in the sense of complete colocalisation as defined in Figure 2a; however, we observed a number of nuclei (25%, see Table 1) in which the barley homologues were in contact at the centromere or at another chromosomal site. This indicates that *Ph1* affects not only centromere structure [10] but also the spatial organization of chromosomes at pre-meiotic interphase. It is, however, impossible to determine from this data whether chromosomes remain unassociated at this stage in the mutant, or whether the barley chromosomes are in fact associated with (unlabelled) wheat homoeologues.

We have shown that homologous chromosome association in polyploid wheat occurs prior to the onset of prophase I. Furthermore, the finding that homologue association occurs in some somatic cell types (tapetum) establishes that this and meiotic division are two clearly separable processes.

Materials and methods

Wheat inflorescences were dissected and immediately fixed in 4% formaldehyde (freshly made from paraformaldehyde) in a PIPES buffer [11]. Individual florets were dissected and sections 100 μm thick were cut under water using a vibratome. The sections were dried down onto multiwell slides coated with glutaraldehyde-activated gamma-aminopropyl triethoxy silane (APTES). Sections were permeabilized by incubation with 2% (w/v) cellulase (Onozuka R-10) in Tris-buffered saline for 1 h at room temperature. *In situ* hybridization and probe preparation were carried out according to the protocols described in [10]. Confocal optical section stacks were collected using a Biorad MRC-1024 UV confocal scanning microscope as described previously [11]. Images were transferred to a Macintosh computer and assembled into composite images using Adobe Photoshop and NIH-Image, a public domain program for the Macintosh written by Wayne Rasband and available via anonymous ftp from zippy.nimh.nih.gov.

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